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A Gene Delivery/Recall System for Neurons Which Utilizes Ribonucleotide Reductase-Negative Herpes Simplex Viruses

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We present data which show that ribonucleotide (RR)-negative herpes simplex virus type-1 (HSV-1) is a useful vector for gene delivery into neuronal cells. For these studies we used hrR3, a genetically engineered HSV-1 mutant which has an in-frame insertion of the bacterial *lacZ* gene into the HSV gene that encodes the large subunit (ICP6) of RR. After infection of rat primary sympathetic neuronal cultures with hrR3, the ICP6::*lacZ* chimeric gene was expressed, as shown by blue staining of the cells upon exposure to X-Gal, a chromogenic β-galactosidase substrate. When the infection was performed in the presence of acyclovir, hrR3 appeared to become "latent"; neither infectious virus nor β-galactosidase activity was detectable in these neuronal cultures at 3 weeks after the acyclovir was removed. However, β-galactosidase activity was inducible in the "latent" cultures by superinfection with ICP6Δ (a RR-negative deletion mutant) without resulting in the "reactivation" of hrR3 and without apparent cytopathic effects. In contrast, superinfection with ICP6Δ + 3.1, a virus derived by marker rescue of ICP6Δ, resulted in the expression of *lacZ*, the release of hrR3 into the culture medium, and cytopathic effects. The introduction of a foreign gene into neuronal cells by a RR-negative herpes simplex virus, and the subsequent induction of gene expression by another noncomplementing virus, may constitute a prototype gene delivery/recall system for neurons.

Herpes simplex virus type-1 (HSV-1) is able to infect both nonneuronal and neuronal cells (*1*). In neurons HSV can either undergo a productive lytic infection or it can enter a state of viral latency. Much is known about the lytic cycle in nonneuronal cells, however, very little is known about HSV infection of neurons, in general, and the course of events that lead to the establishment of latency, in particular. Recently, an *in vitro* model of herpes simplex virus latency was described by Wilcox and Johnson (*2*). This model should facilitate studies of viral–neuronal cell interactions.

It has been suggested that HSV can be used as a vector to introduce genes into neuronal cells (*3, 4*). Based on theoretical considerations, the ideal herpes virus vector would be able to express a foreign gene of interest without causing cytopathic effects. Several types of HSV vectors have been proposed for this purpose. These include defective HSV vectors (*3, 5*) and conditional lethal recombinant viruses which are propagated on helper cell lines (*4*). A similar approach is to utilize mutant viruses that propagate well on virtually all nonneuronal cells, but which are unable to replicate in fully differentiated neuronal cells. HSV encodes a number of genes which are nonessential for growth in nonneuronal cells in tissue culture, but which may be important, or even essential, for growth in postmitotic neurons. Thymidine kinase (tk)-negative mutants, for instance, grow well on fibroblasts in tissue culture as well as at the site of innoculation in mice, but are apparently unable to replicate in mouse neuronal cells (*6, 14*). tk-negative mutants are able, however, to establish a latent infection in mouse ganglia but do not reactivate (*6, 7*). Other mutants which have similar properties include ribonucleotide reductase-negative mutants. HSV encodes a ribonucleotide reductase (RR) consisting of a large subunit (RR1 or ICP6) and a small subunit (RR2). In mice RR-negative HSV mutants have been shown to have reduced virulence (*9*). However, like tk-negative mutants they can establish latency but fail to reactivate (*8*). Cellular RR is present only in actively dividing cells. Neurons, by virtue of their postmitotic state, should have little or no RR activity (*10*). One would predict, therefore, that a RR-negative HSV would find neurons an unsuitable environment for replication. Such mutants, therefore, may have potential as vectors to introduce foreign genes into neurons.

Recently, two genetically engineered RR-negative HSV (KOS) mutants were constructed by Goldstein and Weller (*11, 12*). One mutant, hrR3, has an in-frame in-

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sertion of the bacterial gene *lacZ* into the ICP6 encoding gene (UL39) such that *lacZ* is placed under the control of the ICP6 promoter. Another mutant, ICP6Δ, has a large deletion which removes most of the ICP6 gene. Phenotypically these two mutants behave similarly except that cells infected with hrR3 appear blue after staining with X-Gal, a chromogenic β-galactosidase substrate. The growth of these viruses in actively dividing cells is similar to that of the parental virus, but their growth is severely impaired in growth-arrested, nondividing cells. ICP6Δ+3.1, a virus derived by marker rescue of ICP6Δ, was shown to have the same phenotype as wild-type virus (*12*). We decided to examine the behavior of hrR3 in primary neuronal cultures and to evaluate the expression of *lacZ* in these cells after infection. We present here an interesting preliminary result from these studies.

Primary sympathetic neuronal cultures were prepared from superior cervical ganglia (SCG) of Day-21 embryonic rats as described previously (*2*). The cells were grown in the presence of an antimitotic agent, fluorodeoxyuridine, for 10 days before the experiments were performed. At this time approximately 95% of the cells were neurons, the remaining cells being nondividing Schwann cells and fibroblasts. The cells were infected with hrR3 using 20 plaque-forming units per cell. At various times postinfection the cells were fixed and histochemically stained for β-galactosidase activity. Whereas blue-staining nonneuronal cells appeared at 4 hr, neuronal cells did not stain blue until 16 hr postinfection. By 48 hr approximately 30% of the neurons were stained (Fig. 1). However, the infection of these cells was nonproductive; the amount of hrR3 ($<3.0 \times 10^1$ PFU/ml) detectable in these cultures 3 days after infection was less than the amount detected immediately following infection (1.0×10^2 PFU/ml) (data not shown). In contrast, parallel primary rat SCG cultures which were not pretreated with an antimitotic and which contained numerous dividing nonneuronal cells contained a significant amount of hrR3 (8.7×10^3 PFU/ml) at Day 3 after infection.

We then examined the behavior of hrR3 in long-term cultures of sympathetic neurons using the protocol developed for establishing latency *in vitro* (*2*). At the time of infection, and for 1 week after infection, acyclovir (50 μ*M*) was maintained in the culture medium. The acyclovir was then removed and the cells were cultured for an additional 3 weeks. Under these conditions, no infectious hrR3 was detected in the culture medium (detection limit: 30 PFU/ml) and no β-galactosidase activity was detected histochemically. Treatment of these cultures with anti-nerve growth factor (anti-NGF), which has been shown previously to reactivate latent wild-type virus, had no apparent effect. However, the transcriptionally/translationally quiet ICP6::*lacZ* chimeric gene was induced by superinfection (m.o.i. = 10) of these "latent" cultures with another RR-negative virus, ICP6Δ (Fig. 2B), but no hrR3 (detection limit: 30 PFU/ml) was released into the culture medium and, importantly, the neurons appeared healthy (no granulation of the cell bodies or fragmentation of the neurites were observed). At least four microscopic fields (30–50 neurons per field) were examined per culture. *LacZ* expression was also induced by superinfection (m.o.i. = 10) with a wild-type virus (strain F) and with ICP6Δ + 3.1, a virus derived by marker rescue of ICP6Δ (Figs. 2C and 2D). However, after infection with these viruses, cytopathic effects were evident and hrR3 was detectable (>100 PFU/ml) in the culture medium by the detection of blue-stainable plaques on indicator cells. For unknown reasons the percentage of β-galactosidase expressing cells following superinfection with these three viruses (ICP6Δ, ICP6Δ + 3.1, and wild type) was somewhat variable from experiment to experiment so it was difficult to determine whether there was a difference among the viruses in the efficiency of induction.

It is intriguing that the ICP6::*lacZ* chimeric gene was induced in the "latently" infected hrR3 cultures by superinfection with ICP6Δ. hrR3 and ICP6Δ have mutations which should be noncomplementing and, as one would predict, no infectious hrR3 progeny resulted from this experiment. It is possible that some factor(s) associated with ICP6Δ superinfection can transactivate the ICP6::*lacZ* transcription unit. In this regard, ICP4 would not be a likely candidate as the ICP6 promoter has been shown to be unresponsive to ICP4 (*11*). On the other hand, it was recently demonstrated that the ICP6::*lacZ* chimeric gene could be induced 30- to 40-fold by the virion-associated immediate-early gene transactivator VP16 and 10-fold by ICPO (S. Weller, personal communication). In addition, the promoter for the HSV-2 gene encoding the ribonucleotide reductase large subunit (ICP10) has also been shown to be responsive to VP16 transactivation (*13*). This issue and many others raised by these experiments are currently under investigation.

Our finding that it is possible to deliver a foreign gene into a neuron by an HSV mutant, and to subsequently induce the expression of this gene by a noncomplementing mutant, may constitute a prototype gene delivery/recall system for neuronal cells *in vitro*. Further experiments are necessary to assess the usefulness of this paradigm, or alternative paradigms in which induction does not require superinfection, to realize the potential of this approach to provide controlled expression of foreign genes in healthy neurons.

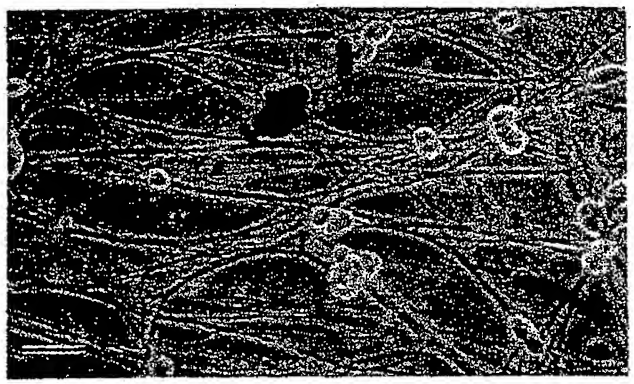

FIG. 1. Infection of a primary rat sympathetic neuron culture with hrR3. Dissociated cells from superior cervical ganglia were grown in the presence of an antimitotic agent, fluorodeoxyuridine, for 10 days before the experiment was initiated. The cells were infected with hrR3 (m.o.i. = 20) and 48 hr after infection the cells were fixed and histochemically stained with X-gal. Arrow indicates a blue degenerating nonneuronal cell. Bar: 50 μm.

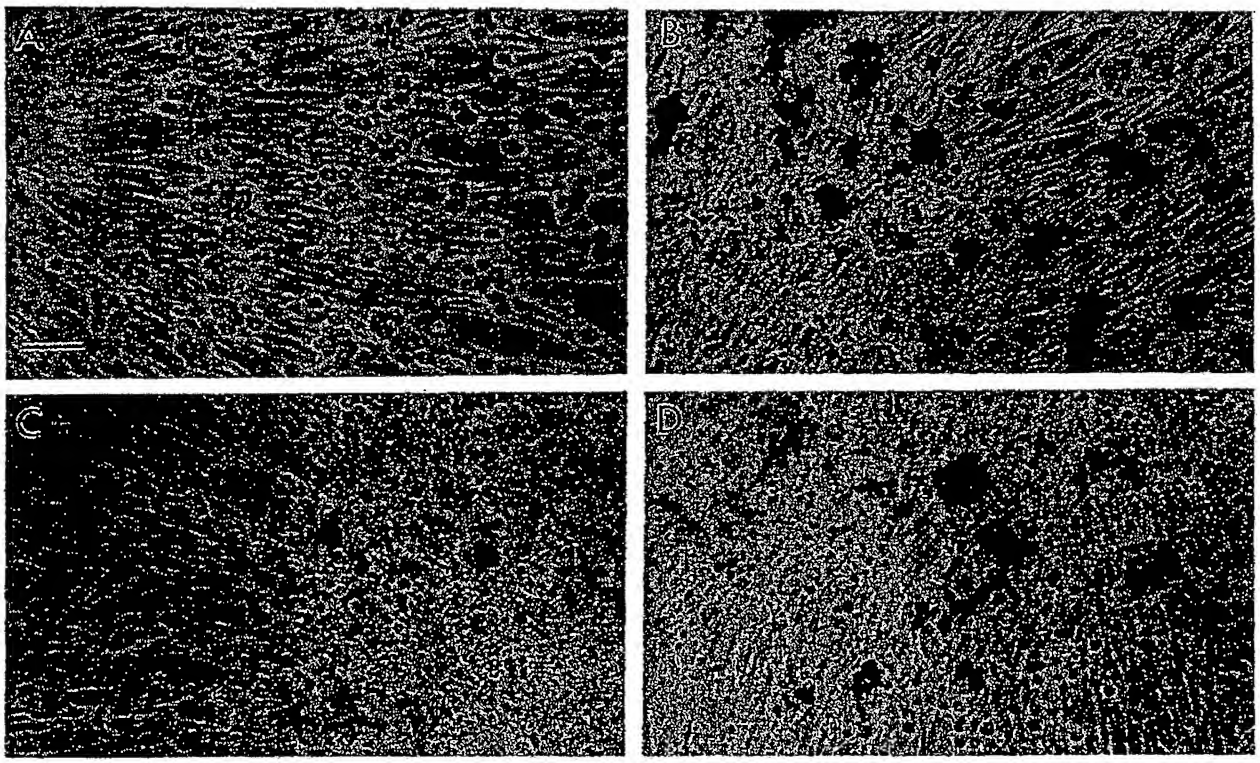

FIG. 2. Induction of expression of *lacZ* in neuronal cultures latently infected with hrR3. A set of SCG cultures was latently infected with hrR3 as described in Ref. (2). Four weeks postinfection, and 3 weeks after removal of the acyclovir, the cultures were treated with: (A) nothing, (B) anti-NGF antibodies plus ICP6Δ superinfection for 4 days, (C) anti-NGF antibodies plus wt HSV (strain F) superinfection for 4 days, or (D) anti-NGF antibodies plus ICP6Δ*3.1 superinfection for 4 days. The cells were then fixed and stained with X-Gal. Bar: 100 μm.

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